(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
6 December 2001 (06.12.2001)

PCT

(10) International Publication Number
WO 01/092463 A3

(51) International Patent Classification[7]: C12N 15/11, 9/00

(21) International Application Number: PCT/IB01/01189

(22) International Filing Date: 30 May 2001 (30.05.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/208,432 31 May 2000 (31.05.2000) US

(71) Applicant *(for all designated States except US)*: **ISTITUTO DI BIOLOGIA CELLULARE, CNR** [IT/IT]; Via E. Ramarini, 32, I-00016 Monterotondo Scalo (IT).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **TOCCHINI-VALENTINI, Glauco, P.** [IT/IT]; Via Cassia, 240, I-00191 Roma (IT). **DEIDDA, Giancarlo** [IT/IT]; Via F. Paolo Bonifacio, 56, I-00156 Roma (IT). **ROSSI, Nicoletta** [IT/IT]; Via P. Togliatti, 1604, I-00155 Roma (IT). **BALDI, Maria, Irene** [IT/IT]; Via di Trasone, 40, I-00199 Roma (IT). **FRUSCOLONI, Paolo** [IT/IT]; Via Monte Pramaggiore, 4, I-00141 Roma (IT).

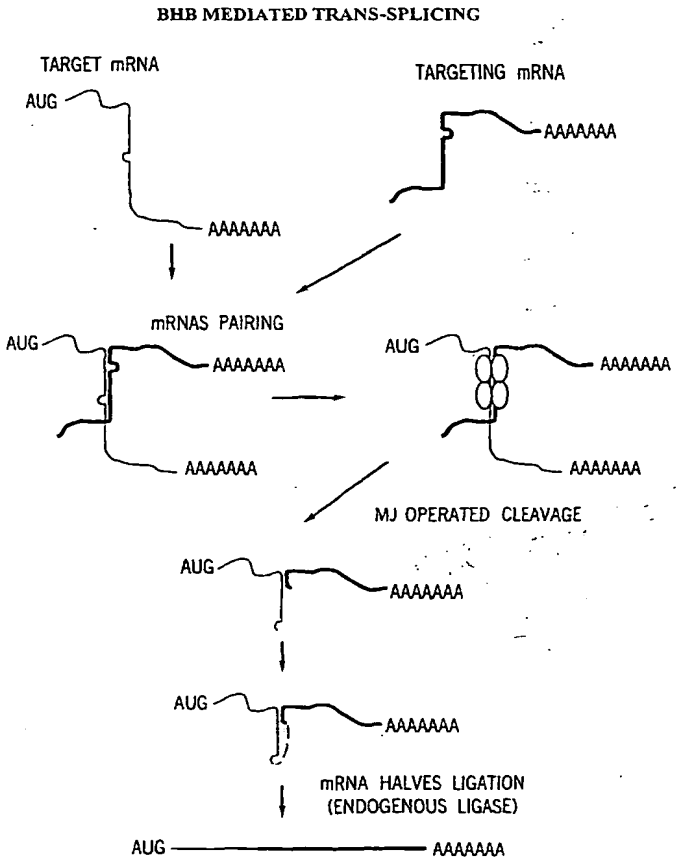
(74) Agent: **DUCKWORTH, Timothy, John**; J.A. Kemp & Co., 14 South Square, Gray's Inn, London WC1R 5JJ (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian

[Continued on next page]

(54) Title: METHOD OF RNA CLEAVAGE



(57) Abstract: A method of cleaving a target RNA molecule is disclosed. In one embodiment the method comprises the step of exposing the target molecule to an eukaryotic tRNA splicing endonuclease, wherein the target molecule is in the bulge-helix-bulge conformation, wherein cleavage occurs within the bulge-helix-bulge and cleavage products are generated, and wherein the target molecule does not comprise a tRNA structure.

WO 01/092463 A3

patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) Date of publication of the international search report:
9 January 2003

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

ational Application No
PCT/IB 01/01189

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/11 C12N9/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, MEDLINE, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FABBRI STEFANIA ET AL: "Conservation of substrate recognition mechanisms by tRNA splicing endonucleases." SCIENCE (WASHINGTON D C), vol. 280, no. 5361, 10 April 1998 (1998-04-10), pages 284-286, XP002216303 ISSN: 0036-8075 cited in the application	1,10
Y	page 285, middle column, line 9 -right-hand column, line 12; figure 2	1-17

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 October 2002	23/10/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Andres, S

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	NASHIMOTO MASAYUKI ET AL: "RNA heptamers that direct RNA cleavage by mammalian tRNA 3' processing endoribonuclease." NUCLEIC ACIDS RESEARCH, vol. 26, no. 11, 1 June 1998 (1998-06-01), pages 2565-2571, XP002216304 ISSN: 0305-1048 the whole document	1-11
Y	LI HONG ET AL: "Crystal structure and evolution of a transfer RNA splicing enzyme." SCIENCE (WASHINGTON D C), vol. 280, no. 5361, 10 April 1998 (1998-04-10), pages 279-284, XP002216305 ISSN: 0036-8075 the whole document	12-17
A	DIENER JOHN L ET AL: "Solution structure of a substrate for the archaeal pre-tRNA splicing endonucleases: The bulge-helix-bulge motif." MOLECULAR CELL, vol. 1, no. 6, May 1998 (1998-05), pages 883-894, XP002216306 ISSN: 1097-2765 cited in the application	
A	WO 95 32283 A (INDIANA UNIVERSITY FOUNDATION) 30 November 1995 (1995-11-30)	
P,X	FRUSCOLONI PAOLO ET AL: "Cleavage of non-tRNA substrates by eukaryal tRNA splicing endonucleases." EMBO REPORTS, vol. 2, no. 3, March 2001 (2001-03), pages 217-221, XP002216307 ISSN: 1469-221X the whole document	1-17

INTERNATIONAL SEARCH REPORT

....ernational application No.
PCT/IB 01/01189

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-9, 12-17 (as far as in vivo methods are concerned) and claim 11 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [X] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-11

   A method for cleaving a target RNA using an eukaryotic tRNA splicing endonuclease.

2. Claims: 12-17

   A method for cleaving a target RNA using an archaeal tRNA splicing endonuclease.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IB 01/01189

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9532283 A	30-11-1995	AU 2601495 A	18-12-1995
		WO 9532283 A1	30-11-1995

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/09, 15/10, 15/11, 15/70, C12P 19/34

A1

(11) International Publication Number: WO 95/32283

(43) International Publication Date: 30 November 1995 (30.11.95)

(21) International Application Number: PCT/US95/06519

(22) International Filing Date: 23 May 1995 (23.05.95)

(30) Priority Data:
08/247,776 23 May 1994 (23.05.94) US

(71) Applicant: INDIANA UNIVERSITY FOUNDATION [US/US]; Showalter House, P.O. Box 500, Bloomington, IN 47401 (US).

(72) Inventors: FRANK, Daniel, H.; Apartment 41, 1600 East Hillside Drive, Bloomington, IN 47401 (US). HARRIS, Michael, E.; 446 Poplar Drive, Ellettsville, IN 47429 (US). PACE, Norman, R.; 1009 South High Street, Bloomington, IN 47401 (US).

(74) Agents: GANDY, Kenneth, A. et al.; Woodard, Emhardt, Naughton, Moriarty & McNett, Bank One Center/Tower, Suite 3700, 111 Monument Circle, Indianapolis, IN 46204 (US).

(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TM, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
With international search report.
With amended claims.

(54) Title: SEQUENCE-SPECIFIC ENDONUCLEASES, AND PREPARATION AND USE OF SAME

(57) Abstract

RNase P RNA conjugates which function as sequence-specific endonucleases are described. Preferred conjugates include a covalently-attached Internal Guide Sequence that binds exogenous RNA substrates via Watson-Crick basepairing. This, in effect, converts a structure-specific ribozyme into a sequence-specific ribozyme useful *inter alia* in antiviral and other therapies.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

SEQUENCE-SPECIFIC ENDONUCLEASES, AND PREPARATION AND USE OF SAME

BACKGROUND OF THE INVENTION

The present invention relates generally to the biotechnological arts, and in particular to RNAs which function as enzymes, i.e. ribozymes, which demonstrate sequence-specific endonuclease activity.

Ribonuclease P (RNase P) is a known enzyme which removes 5' leader sequences from pre-tRNAs to form mature tRNAs. RNase P has been identified in organisms representing all three primary phylogenetic domains; Bacteria, Archaea and Eucarya (Woese, 1990). RNase P enzymes are known to function *in vivo* as holoenzymes composed of single protein and RNA subunits (Pace and Smith, 1990; Altman et al., 1993). The RNA subunit of bacterial RNase P has been shown to function catalytically *in vitro* in the absence of the protein subunit and thus represents an RNA enzyme, or ribozyme (Guerrier-Takada et al., 1983). Unlike all other characterized ribozymes (i.e. groups I and II autocatalytic introns, hammerhead, hairpin and hepatitis delta virus), RNase P acts *in trans* in its native form to carry out multiple-turnover reactions. Furthermore, RNase P binds pre-tRNAs by interactions with elements of tertiary structure, rather than by extensive Watson-Crick base-pairing. Thus, the native RNase P ribozyme mainly recognizes substrate structure instead of

sequence per se.

The inter-molecular nature of the RNase P reaction has presented several challenges to the study of RNase P structure and function. One consideration is that the rate-limiting step in pre-tRNA cleavage is the release of product, rather than the chemical step (i.e. bond breaking and making), under substrate-saturated conditions (Reich et al., 1988; Tallsjî and Kirsebom, 1993). This complicates kinetic and mutational analyses of RNase P-mediated catalysis because multiple-turnover reaction rates are dominated by substrate binding and product release rather than the chemical process. Although reaction conditions have been described in which chemistry is rate-limiting (e.g. in the presence of Ca2+ instead of Mg2+, or at low pH; Smith and Pace, 1993), these non-native conditions are less than ideal for the study of RNase P enzymology. Another challenge to the study of RNase P is that it is not altered by the cleavage reaction. Consequently, potentially informative strategies such as *in vitro* selection are not applicable to this system because they require separation of functional and non-functional pools of molecules (Gold et al., 1993; Szostak and Ellington, 1993).

Researchers have avoided the constraints imposed by this inter-molecular reaction by covalently linking the RNase P RNA to its pre-tRNA substrate, thus creating a self-cleaving ribozyme (Kikuchi et al., 1993). When pre-tRNA is linked to either the 5' or 3' terminus of RNase P, intra-molecular cleavage can be achieved, but it is both inefficient and inaccurate. Indeed, inter-molecular cleavage, rather

than true self-cleavage, prevails under most reaction conditions when the pre-tRNA is linked to the native termini of RNase P (Kikuchi et al., 1993).

To facilitate the efficiency and accuracy of intra-molecular cleavage, the applicants constructed ribozyme-substrate conjugates in which the pre-tRNA substrate was positioned at the active-site of RNase P. The secondary structure of bacterial RNase P RNA is well-established (James et al., 1988; Brown and Pace, 1992; Haas et al., 1994). Moreover, intermolecular crosslinking and chemical footprinting studies have identified regions of RNase P RNA that are in close proximity to specific nucleotides in the bound substrate pre-tRNA (Burgin and Pace, 1990; Nolan et al., 1993). The applicants achieved specific positioning of the pre-tRNA substrate within a conjugate by fusing a pre-tRNA gene to a circularly permuted RNase P gene. The circularly permuted RNA was created by rearranging the RNase P RNA gene so that new 5' and 3' ends were formed at adjacent nucleotides within the RNase P RNA primary structure. Thus, novel 5' and 3' ends can be created at any pair of nucleotides within the RNase P RNA and the tRNA gene can be appended to either end of the resulting molecule. Kinetic analyses demonstrate that disruption of the phosphodiester backbone of RNase P usually does not alter enzymatic activity to a significant extent (Reich et al., 1986; Guerrier-Takada and Altman, 1992; Waugh and Pace, 1993), so there is considerable flexibility in the design of tethered molecules while maintaining enzymatic activity.

SUMMARY OF THE INVENTION

In their study of RNase P conjugates, the applicants discovered that it is possible to incorporate into the oligoribonucleotide to which the RNase P is covalently bonded a sequence which hybridizes with a target RNA, so as to confer sequence-specific endonuclease activity to the RNase P conjugate. One preferred embodiment of the invention therefore provides an endonuclease for sequence-specific cleavage of a target nucleotide molecule such as an RNA. The endonuclease includes an RNA molecule having nucleotides which provide the enzymatic activity of Ribonuclease P, and an oligonucleotide covalently bonded to the RNA molecule to form a conjugate. The oligonucleotide includes a predetermined nucleotide sequence complementary to and available for hybridization with a nucleotide sequence of the target nucleotide molecule, wherein the conjugate is an endonuclease which cleaves the target molecule in a sequence-specific fashion.

Another preferred embodiment of the invention provides a method for sequence-specific intermolecular cleavage of a target nucleotide molecule such as an RNA. The method includes reacting the target molcule with a sequence-specific endonuclease including an RNA molecule having nucleotides which provide the enzymatic activity of Ribonuclease P, and an oligonucleotide covalently bonded to the RNA molecule to form a conjugate. The oligonucleotide includes a predetermined nucleotide sequence complementary to and available for hybridization with a nucleotide sequence of the target molecule. The conjugate cleaves the target molecule in a sequence-specific fashion.

Still further preferred embodiments of the invention provide methods for making sequence-specific endonucleases, genes encoding the sequence-specific endonucleases, and vectors incorporating such genes.

The conjugates, or sequence-specific endonucleases, of the invention will find use in gene therapy, for example by the selective degradation of pathogenic RNAs (e.g. degradation of viral transcripts or RNA genomes in antiviral therapies) or of disease-causing mRNAs (e.g. the degradation of RNA products of oncogenes). The sequence-specific endonucleases of the invention will also find use as reagents for inhibiting the expression of the specific genes, for example knocking out the expression of developmental genes, and will also be useful as reagents for sequence-specific cleavage of RNA *in vitro* which can be used in the detection of virus in tissue and fluid samples.

Additional embodiments, features and advantages of the invention will be apparent from the following description.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 shows the sequences and secondary structures of native RNase P and of tethered pre-tRNA-RNase P conjugates studied in the Experimental. Arrows denote predicted sites of endonucleolytic cleavage.

Fig. 2 shows the results of cleavage assays using the tethered pre-tRNA-RNase P conjugates.

Fig. 3 shows the results of primer extension analyses which demonstrate the accuracy of cleavage exhibited in the assays shown in Fig. 2.

Fig. 4 shows the inhibition of cleavage of tethered pre-tRNA-RNase P conjugates by competitor tRNA.

Fig. 5 illustrates the design of sequence-specific RNase P endonucleases in accordance with the invention.

Fig. 6 illustrates the sequence-specific inter-molecular cleavage of target RNAs by sequence-specific endonucleases of the invention.

DESCRIPTION

For the purposes of promoting an understanding of the principles of the invention, reference will now be made to certain embodiments thereof and specific language will be used to describe the same. It will nevertheless be understood that no limitation of the scope of the invention is thereby intended, such alterations, further modifications and applications of the principles of the invention as described herein being contemplated as would normally occur to one skilled in the art to which the invention relates.

A feature of the present invention is the discovery that RNase P can be modified to form a sequence-specific endonucleases capable of cleaving an exogenous or separate target nucleotide sequence in a defined, sequence-specific manner. The modification can be achieved by attaching an oligonucleotide to the RNase P RNA molecule to form a conjugate, wherein the oligonucleotide includes a region which hybridizes with the target sequence. This hybridization with the target substrate promotes the sequence-specific cleavage of the substrate by the conjugate (i.e. the conjugate functions as a sequence-specific endonuclease). The sequence-specific endonucleases of the invention will thus find use in gene therapy, for example by selective dedregation of pathogenic RNAs and (e.g. viral transcripts or RNA genomes) or of disease-causing mRNAs (e.g. products of oncogenes. The sequence-specific endonucleases of the invention will also find use as reagents for inhibiting the expression of the specific genes, for example knocking out the

expression of developmental genes, and will also be useful as reagents for defined, sequence-specific cleavage of RNA *in vitro*.

RNase P

RNase P is present in the cells of organisms representing all three kingdoms. Both prokaryotic and eukaryotic RNase P enzymes have been identified and their activities characterized. The sequences of both prokaryotic and eukaryotic RNase P enzymes, including human RNase P, are known and their secondary structures have been identified. In general, studies have shown that the sequences, structures and activities of prokaryotic and eukaryotic RNase P enzymes are very similar. *In vivo*, RNase P functions as a holoenzyme composed of single protein and RNA subunits. The RNA subunit of bacterial RNase P functions catalytically in the absence of any protein subunit and thus represents an RNA enzyme, or "ribozyme". Because this RNA subunit is itself catalytic, the use of the holoenzyme is not necessary to broader aspects of the present invention. That is, the RNA subunit, or RNase P ribozyme, can be used alone. In addition, it is well known that nucleotide sequences representing fragments or variants (i.e. containing nucleotide deletions or substitutions) of ribozymes such as RNase P ribozymes, nevertheless retain the enzymatic function of the parent ribozyme (Waugh et al., 1989). Thus, as used herein, the term RNase P ribozyme is intended to encompass not only the complete RNase P nucleotide sequence but also active fragments or variants thereof which exhibit a similar ability to enzymatically cleave RNAs.

Oligonucleotide

In accordance with the invention, a native RNase P ribozyme is modified to incorporate a covalently attached oligonucleotide, such as an oligoribonucleotide, which contains a nucleotide sequence capable of hybridizing with a nucleotide sequence in a target molecule such as an RNA, thus forming a conjugate exhibiting sequence-specific endonuclease activity and capable of cleaving the target molecule in a sequence-specific fashion. Herein, that portion of the covalently-attached oligonucleotide sequence which forms the hybridization element will be referred to as the "Internal Guide Sequence" (IGS). The oligonucleotide can be of any suitable length to accommodate the IGS and achieve the desired sequence specificity, and in practice will typically contain at least 3 nucleotides up to several thousand (e.g. 5000) or more nucleotides, more typically in the range of about 3 to 7 up to about 100 nucleotides. The length and extent of complementarity of the IGS necessary for sufficient hybridization or base pairing with the target will also vary with the circumstances at hand and will be readily determined by those practiced in the area. Preferably, the IGS will include at least 3 nucleotides, up to about 5000 or more, and more preferably will include at least 5 nucleotides, typically falling within the range of about 5 to about 100 nucleotides. The IGS can comprise the full length of the oligonucleotide or only a portion thereof. As well, the IGS can include intermittent complementary and non-complementary regions so long as the IGS sufficiently binds the target to enable sequence-specific cleavage by the conjugate.

Preparation of RNase P Conjugates

Preferred RNase P conjugates are constructed so that the oligonucleotide containing the IGS is attached to the RNase P RNA at a location other than the 5' or 3' end of the native molecule, more preferably attached at or in close proximity to the active-site of the RNase P enzyme. Candidate locations for attachment of the oligoribonucleotide at or near the active site are identifiable, for example, as regions at or near to specific nucleotides in bound substrates such as pre-tRNAs. In the preferred method of making the inventive conjugates, specific positioning of the IGS-containing oligonucleotide sequence within a conjugate is achieved by fusing a gene encoding the oligonucleotide sequence to a circularly permuted RNase P ribozyme gene. The circularly permuted RNA can be created by rearranging the RNase P ribozyme gene so that new 5' and 3' ends are formed at adjacent nucleotides within the RNase P ribozyme primary structure. Thus, novel 5' and 3' ends can be created at any pair of nucleotides within the RNase P ribozyme RNA and the gene encoding the oligonucleotide can be appended to either end of the resulting molecule. Kinetic analyses have demonstrated that disruption of the phosphodiester backbone of RNase P usually does not alter enzymatic activity to a significant extent (Reich et al., 1986; Guerrier-Takada and Altman, 1992; Waugh and Pace, 1993), so there is considerable flexibility in the design of conjugate molecules while maintaining enzymatic activity necessary for the desired endonuclease function.

Vectors Containing Genes Encoding Conjugates

Genes encoding conjugates of the invention can be suitably incorporated into vectors for transcription *in vitro* or *in vivo*. The vectors can also include a promoter for an RNA polymerase, e.g. the phage T7 RNA polymerase promoter, as well as a gene encoding a suitable RNA polymerase which, upon expression, provides RNA polymerase which transcribes the conjugate genes to afford conjugates with the desired endonuclease activity. Vectors of the invention for *in vivo* expression or transcription will be suited for use with the selected host, for instance a bacterial or mammalian (including human) host. Suitable vectors will include, for example, plasmid or viral vectors such as adenoviral vectors. Vectors for *in vitro* synthesis of the inventive conjugates will preferably include the conjugate gene fused to a suitable RNA polymerase promoter, whereby run-off transcription using an RNA polymerase is enabled, as described in more detail in the Experimental below.

Use of RNase P Conjugates

RNase P conjugates of the invention possessing sequence-specific endonuclease activity can be engineered to cleave a wide variety of target RNAs whose sequences are known or at least partially known. The inventive conjugates will have therapeutic utility in man and in other animals and plants. For example, they will be useful in gene therapy by selective degradation of pathogenic RNAs such as viral transcripts or RNA genomes, or of disease-causing mRNAs such as products of oncogenes. Active conjugates of the invention can be administered directly in a suitable pharmaceutical vehicle, or genes encoding active conjugates and containing appropriate regulatory elements for expression can be incorporated into the host genome to provide expression of active conjugates in the host and cleavage of the target RNAs. The sequence-specific endonucleases of the invention will also be useful as reagents for defined, sequence-specific cleavage of RNA *in vitro*, for example in the study of viral activity and/or for inhibiting or knocking out the expression of specific genes, for example developmental genes. Representative viral pathogens to which conjugates of the present invention have application include, for example, the herpes simplex virus (HSV) and the AIDS virus (HIV). Conjugates of the invention may also find use in the study, sequencing and manipulation of known and unknown RNAs in a fashion similar to the long-standing use of endonucleases in the study, sequencing and manipulation of known and unknown DNAs. In addition, RNase P RNA has been shown capable of cleaving single-stranded DNAs, and thus conjugates of the invention may also find use in a variety of settings, including in applications similar to those above but

in which cleavage of DNA is desired.

Conjugates of the present invention will provide many advantages in therapeutic use. For example, cleavage mediated by the conjugates is not limited to a specific sub-cellular locale since it is independent of native RNase P activity; the IGS-containing oligoribonucleotide carries the ribozyme. Thus a wide range of RNA substrates, such as pre-mRNA and mRNA, can be targeted for destruction *in vivo*.

Advantages relative to toxicity are also provided by the inventive endonucleases. The preferred inventive conjugates, while being derivatives of the RNase P RNA, have a substrate specificity which has been modified to an extent that the enzyme no longer interacts with exogenous pre-tRNA. Expression or other use of the inventive conjugates *in vivo* should not, therefore, interfere with normal RNase P activity. Further, the preferred conjugates carry out a novel function that is independent of the normal cellular processes mediated by RNase P. Individual conjugate genes can thus be substantially modified, through *in vitro* or *in vivo* selection protocols, to "tune" a given conjugate enzyme to a particular task. For example, conjugates could be engineered to function under specified conditions such as high temperatures, low ionic strength, or other pertinent conditions.

The following experimental is given in order to illustrate the invention and its preferred features and advantages. It will be understood that this experimental is illustrative and not limiting in nature.

EXPERIMENTAL

A. MATERIALS AND METHODS

Oligonucleotides.

PT292Forward: CGG GAT CCG GTA GGC TGC TTG AGC CAG T

PT292Reverse: GAA GAT CTA CGG GTT CAG TAC GGG CCG T

ECG332Forward: TAA TAC GAC TCA CTA TAG AAT GAC TGT CCA CGA CAG

ECG332Reverse: ATC TAG GCC AGC AAT CG

PRE.1: GAT CCT ACC GGA CCA TTT TGG GTA C

ANTI-PRE.1: CCA AAA TGG TCC GGT AG

PRE.2: GAT CCT GAA CTA CCG GAC CAT TTT GGG TAC

ANTI-PRE.2: CCA AAA TGG TCC GGT AGT TCA G

ENDO.1: CGG AAT TCT AAT ACG ACT CAC TAT AGT TCA GTT GGT TAG AAT GCC

ENDO.2: CGG AAT TCT AAT ACG ACT CAC TAT AGT TGG TTA GAA TGC CTG CC

52R: CCG CGA CCT CCT GCG TG

Plasmids.

The following plasmids were used in this work: pUC18 was purchased from New England Biolabs. pDW98, pDW128 and pDW152 were provided by D. Waugh (1989). pDS153F was provided by D. Smith pDS153F is a derivative of pDW 152 in which a FokI endonuclease site was introduced immediately 3' of the RNase P RNA gene for the purpose of generating the prices 3' end of RNase P during *in vitro* transcription. pBST-mBSD-CCA and p153Bsttan were provided by J. Nolan (1993).

Preparation of RNAs

Circularly permuted RNAs (CP292 and CP332) were generated using techniques essentially as described by Nolan et al., 1993. Template DNAs for *in vitro* transcription were synthesized by PCR from tandemly

repeated genes. The two primers define the endpoints of the PCR product and thus the 5' and 3' ends of the transcribed RNA. The upstream primer contained the core promoter for phage T7 RNA polymerase. PCR reactions contained 30mM Tris-HCl, pH 8.3, 1mM dNTPs, 50mM potassium chloride, 1.5mM magnesium chloride, 0.05% NP 40, 1-10pg tandem RNase P RNA or tRNA genes and 100pmoles of the forward and reverse primers.

Cloning.

The construction of circularly permuted RNase P RNA genes CP292 and CP332 is described above. Plasmid pTP5 was constructed by inserting the Bam HI/Xho I fragment of DW98 that carries the native RNase P gene into the Bam HI/Sal I sites of pDW152. Plasmid pTP292 was constructed by inserting a Bam HI/Bgl II digested Polymerase Chain Reaction (PCR) fragment, which carries CP292, into the Bam HI site of pDS153F. Plasmid pPT332 was constructed by first inserting a PCR fragment carrying CP332 into the Sma I site of pUC18 to create pMEH1. The Kpn I/Nlu I fragment of pDW128, which carries the pre-tRNA^{Asp} gene, was subsequently ligated into the Kpn I and Nar I sites of pMEH1 to create pPT332.

Endo.P1 and Endo.P2 genes were synthesized via PCR of pTP292 with oligos ENDO.1 or ENDO.2 as forward primers and PT292Reverse as the reverse primer. PCR fragments were digested with Eco RI and Bgl II then inserted into the Eco RI and Bam HI sites of plasmid p153Bsttan to create pENDO.1 and pENDO.2. Substrate RNA genes were constructed by ligating annealed pairs of oligonucleotides (PRE.1 + ANTI-PRE.1 or PRE.2 + ANTI-PRE.2) encoding the precursor sequences into the Kpn I and Bam HI sites of plasmid pDS153F to create

plasmids pPRE.1 and pPRE.2. Oligonucleotides were annealed and subsequently purified following the protocol of Frank et al. (1994).

Preparation of RNA transcripts.

RNAs were synthesized *in vitro* by run-off transcription using T7 RNA polymerase. Transcription reactions followed the protocol of Milligan and Uhlenbeck (1989) except that the (NTP) concentration was raised to 5 mM. RNAs were internally radiolabeled by addition of 50µCi of [alpha-32P]GTP to the transcription mix; the concentration of unlabeled GTP was lowered to 0.2 mM in these reactions.

Transcription reactions of tethered RNAs were carried out at 25°C, rather than 37°C, to minimize self-cleavage. Transcription products were phenol-chloroform extracted twice, ethanol precipitated and loaded onto a 6% polyacrylamide/7M urea gel. Following gel electrophoresis, RNAs were visualized by either U.V. shadow or autoradiography. The appropriately sized bands were excised and RNA eluted from the gel slices by passive diffusion (overnight, 25°C) into a buffer consisting of 0.3 M sodium acetate, 10 mM Tris-Cl (pH 7.4), 1 mM EDTA, 0.5 % SDS. The RNA eluate was extracted once with phenol-chloroform and once with chloroform before ethanol precipitation. Pelleted RNA was resuspended in H_2O and quantitated by either U.V. absorbance (260 nm) or specific radioactivity.

RNAs were synthesized from the following linearized plasmids (the restriction enzyme used to digest each plasmid is listed parenthetically): 1)

pre-tRNAAsp - pDW152 (Bst NI); 2) native RNase P RNA - pDW98 (Sna BI); 3) mature tRNAAsp - pBST-mBSD-CCA (Bst NI); 4) TP5 - pTP5 (Fok I); 5) TP292 - pTP292 (Fok I); 6) PT332 - pPT332 (Bst NI); 7) Endo.P1 - pENDO.1 (Fok I); 8) Endo.P2 - pENDO.2 (Fok I); 9) Pre.1 - pPRE.1 (Fok I); 10) Pre.2 - pPRE.2 (Fok I).

Cleavage Assays.

Unless otherwise noted, all cleavage reactions were conducted in a buffer of 16.5 mM PIPES, 44 mM Tris-Cl (pH 8.0), 0.1% SDS, 3 M ammonium acetate and 25 mM magnesium chloride (Smith and Pace, 1993). To accurately measure rate constants for the TP292 and PT332 reactions, cleavage reactions were carried out at pH 6.0 and sodium chloride was substituted for ammonium acetate (Smith and Pace, 1993). For the dilution experiments (Fig. 2 (Nat. P: native RNase P reaction), Table), equivalent quantities of enzyme were diluted into either 4 µl or 400 µl reaction mixes to give final RNA concentrations of 2 nM or 0.02 nM, respectively (substrate was similarly diluted in the native RNase P reactions). In the tRNA inhibition experiments, RNA concentrations were as follows: 2 nM enzyme, 2 nM substrate (for the native RNase P reaction) and 50 nM, 250 nM or 1250 nM unlabeled competitor tRNA. Conjugate reactions employed 100 nM enzyme and 100 nM substrate.

In all cleavage reactions, enzyme and substrate RNAs were mixed in reaction buffer (minus magnesium chloride) on ice, heated for five minutes at 65°C and then shifted to the reaction temperature, 50°C. Reactions were initiated by the addition of pre-warmed magnesium chloride and quenched, after either 60 min. (native RNase P and TP5) or 15 min. (TP292 and PT332), by addition of 3 volumes ice-cold

ethanol, 1-2 volumes 50 mM EDTA and 4 µg glycogen. Following precipitation, precursor and product RNAs were resolved by electrophoresis through denaturing 6% polyacrylamide/7M urea gels. Precursor and product RNA levels were quantitated by phosphorimager analysis (Molecular Dynamics) of dried gels.

Reaction rate constants for the TP5, TP292, PT332 and native RNase P reactions were obtained by measuring the extent of cleavage as a function of time. The apparent rate constant, k^{app}, is given by the slope of a plot of ln(So/St) versus time, where So equals the initial substrate concentration and St equals the substrate concentration at a given time-point (Segal, 1975). Three to four separate cleavage time-courses were performed for each enzyme dilution and slopes were calculated by linear regression.

Assessment of Cleavage Accuracy.

TP292 and PT332 large-scale (0.4 ml) cleavage reactions were performed as described above with a ribozyme concentration of 1 nM. For the large-scale native RNase P reaction the ammonium acetate concentration was lowered to 1 M and the reaction was incubated at 37°C for 1 hr. Final enzyme and substrate concentrations were 10 nM. Reactions were quenched by ethanol precipitation. Primer extension analysis of reaction products followed the protocol of Burgin et al. (1990) and used the $tRNA^{Asp}$-specific oligonucleotide 52R (Nolan et al., 1993). The results are shown in Fig. 3, in which the following desigations are used: predicted cleavage sites are marked by closed circles; G,A,T,C designate RNA sequencing lanes; + designates cleaved RNA; - designates uncleaved RNA.

Terminal nucleotide analysis was also performed. Aliquots of the large-scale reactions were labeled with [5'-32P] cytidine bisphosphate (pCp) and T4 RNA ligase (England et al., 1980) and the 5' products of the cleavage reactions were gel-purified. RNAs were hydrolyzed with 0.04 N NaOH (37°C overnight), neutralized with HCl and the resulting 3' nucleoside monophosphates were resolved by two-dimensional thin-layer chromatography on 13254 cellulose (Kodak) following the protocol of Nishimura (1979). The solvents used in chromatography were as follows: 1) first dimension: 5:3 isobutyric acid: 0.5 M NaOH; 2) second dimension: 70:15:15 isopropanol: HCl: H20. The migration of unlabeled 3' nucleoside monophosphates, added as markers, was monitored by U.V. shadow.

B. RESULTS

Tethered pre-tRNA-RNase P Molecules.

The pre-tRNA-RNase P RNA conjugates examined in this study are shown in Figure 1 (see Materials and Methods for details of constructions). All tethered molecules comprised *E. coli* RNase P RNA joined to *B. subtilis* pre-tRNA^{Asp}. In construct TP5, pre-tRNA was linked to nucleotide 5 of RNase P RNA (As used herein "PT" indicates a tRNA linked to the 3' end of RNase P, while "TP" indicates a tRNA linked to the 5' end of RNase P; the numeral specifies the native nucleotide at the 5' end of the circularly permuted RNase P). Since the 5' and 3' ends of RNase P RNA are remote from the active-site it was expected that tight linkage of substrate to the 5' end of RNase P would not result in efficient self-cleavage (Kikuchi

et al., 1993). TP5 is thus a negative control for comparison with conjugates tethered at the active-site. The rationale for the design of two active-site-tethered molecules, TP292 and PT332, is as follows:

Nucleotide G292 of RNase P was selected as a conjugation site for the 3' terminus of pre-tRNA because this nucleotide is adjacent to the 3' end of tRNA in the native ribozyme-substrate complex. Indeed, G292 is crosslinked by pre-tRNA modified with a 3' photoaffinity agent. In addition, pre-tRNA protects G292 from chemical modification (Knap et al., 1990) and this protection requires the presence of the tRNA 3' terminal CCA sequence. TP292 was constructed by fusing the 3' end of pre-tRNA, by a six nucleotide linker, to the 5' end of a circularly permuted RNase P gene that begins at position G292 (Fig. 1).

The design of PT332 RNA was based on similar criteria. Mature tRNA carrying a crosslinking agent attached to its 5' phosphate, the phosphate acted upon by RNase P, crosslinks to two regions of RNase P RNA, one of which includes nucleotide G332 (Burgin and Pace, 1990). In addition, bound pre-tRNA specifically protects G332 of RNase P RNA from chemical modification, but mature tRNA does not, indicating that the 5' leader sequence of pre-tRNA is juxtaposed to G332 in the native enzyme-substrate complex. Therefore, PT332 was fashioned by linking pre-tRNA sequences to a circularly permuted RNase P RNA that begins at position G332. A thirteen-nucleotide linker separates the cleavage site in tRNA and RNase P sequences (Fig. 1).

Initial Characterization of Cleavage Reactions.

Tethered RNAs were synthesized by in vitro run-off transcription using T7 RNA polymerase and purified by denaturing gel electrophoresis (Milligan and Uhlenbeck, 1989). In all experiments, RNAs were heated to 65°C in reaction buffer lacking Mg2+ for five minutes to allow renaturation, then shifted to reaction temperature for five minutes before addition of Mg2+ to initiate the reaction.

One hallmark of a self-cleavage reaction is that the rate of reaction is expected to be independent of enzyme concentration. In contrast, the rate of an inter-molecular cleavage reaction is expected to be proportional to enzyme concentration at sub-saturating substrate concentration. Figure 2 shows a gel analysis of the reaction products of native and tethered RNase P RNAs at different RNA concentrations. At a concentration of 2 nM, all tethered RNAs undergo cleavage to varying extents (Fig. 2; note that in Fig. 2, TP292 and PT332 molecules were reacted for 15 min. while native RNase P and TP5 were reacted for 60 min.). At 0.02 nM, however, both the native RNase P and TP5 reaction rates are significantly decreased, whereas the extents of the TP292 and PT332 reactions are unaffected. This suggests (but does not prove, see below) that TP292 and PT332 RNAs mainly cleave intra-molecularly.

The accuracy of cleavage by the tethered RNAs can be judged by comparison of the reaction products to those of the standard inter-molecular reaction, in which pre-tRNA is cleaved to form a 77 nucleotide

mature tRNA and a 33 nucleotide 5' leader sequence. As predicted, cleavage of RNAs TP5 and TP292 produces a fragment that comigrates with the 5' leader sequence and a product that runs just below the uncleaved precursor. PT332 also generates the products expected from use of the authentic cleavage site: mature tRNA (77 nt.) and a longer fragment corresponding to RNase P. The multiple mature tRNA fragments produced in the PT332 cleavage reaction are due to 3' terminal heterogeneity in the precursor, generated during *in vitro* transcription (Milligan and Uhlenbeck, 1989), rather than mis-cleavage (below).

The optimal reaction conditions for both TP292 and PT332 were found to be 2.5 - 3.0 M ammonium acetate and 25 mM magnesium chloride with incubation at 50°C. Omission of the 65°C pre-incubation step dramatically decreased cleavage efficiency. Under these conditions, typically 60-90% of input TP292 or PT332 RNAs are rapidly cleaved (within 15 minutes), whereas the extent and rate of the TP5 cleavage reaction are significantly impaired (see below). The requirement for high ionic strength for efficient cleavage by TP292 and PT332 may reflect increased structural flexibility at low ionic strength, a consequence of interrupting the core structure of the ribozyme by insertion of the substrate. Circularly permuted RNase P RNAs commonly require higher ionic strength for optimal activity than does the native RNA. High monovalent cation concentrations presumably decrease unfavorable, repulsive ionic forces and/or increase stabilizing hydrophobic interactions such as base stacking. The requirement of TP292 and PT332 for higher temperature than optimal for the native RNase P reaction may indicate a tendency of PT332 and TP292 to form alternative,

non-catalytic structures; elevated temperature may promote formation of the native (catalytic) form of the enzyme-substrate complex.

TP292 and PT332 Cleavage is Accurate.

The cleavage sites used by TP292 and PT332 were mapped more precisely by primer-extension analysis of 3' products and 3'-terminal nucleotide analysis of 5' products. Figure 3a shows primer-extension analyses of the cleavage products of unlabeled native RNase P, TP292 and PT332 RNAs using oligonucleotide primers complementary to tRNA. Run-off transcripts map the 5' end(s) of the processed tRNA molecules and correspond to the authentic RNase P cleavage site for both TP292 and PT332. Minor extension products one nucleotide longer than the prominent band were seen in all products. This could be due to a low frequency of mis-cleavage or, more likely, to the non-specific addition by reverse transcriptase of non-encoded nucleotides to the 3' ends of run-off transcripts (Clark, 1988). Mis-cleavage that results in a longer 3' product predicts a similarly shortened 5' product, an hypothesis that was tested by 3'-terminal nucleotide analysis. The 5' products that result from cleavage of TP292 (i.e. 5' leader) and PT332 (i.e. cleaved RNase P) were isolated and the 3' terminal nucleotide of each product determined (described in Materials and Methods). More than 99% of the 3' termini of both samples corresponded to the nucleotide (U) expected for cleavage at the authentic RNase P cleavage site (Fig. 3B). Both TP292 and PT332 thus exhibit precise cleavage of the appropriate phosphodiester bond.

Active-Site-Tethered RNAs Efficiently Self-cleave.

The results presented in Figure 2 suggest that TP292 and PT332 undergo self-cleavage. To more rigorously address the question of self-cleavage by TP292 and PT332, a determination was made of the effect of dilution upon the apparent reaction rate constant, k_{app}, for each of these conjugates, as well as for TP5 and the native reaction. Again, in an intra-molecular reaction k_{app} is expected to be insensitive to dilution of reactants. Under the optimal reaction conditions the rates of TP292 and PT332 cleavage were too fast to measure accurately; assays with these RNAs were performed, therefore, under conditions known to slow the native RNase P RNA chemical reaction rate. Previous analysis of the native RNase P reaction showed that reducing the pH from 8.0 to 6.0 causes an approximately 100-fold reduction in the rate of catalysis in single-turnover reactions (Smith and Pace, 1993); indeed, under low pH conditions it was possible to determine the rates of the TP292 and PT332 reactions. The apparent rate constants, k_{app}, for TP5, TP292, PT332 and native RNase P, at 2 nM and 0.02 nM enzyme (and substrate), are summarized in the Table. The rate constants for both TP292 and PT332 are independent of RNA concentration, within the range tested. The insensitivity of k_{app} to concentration of reactants clearly demonstrates that the TP292 and PT332 tethered molecules undergo self-cleavage. In contrast, both the native RNase P and TP5 reaction rates are significantly decreased upon 100-fold dilution of the ribozymes.

As a final test of self-cleavage, native RNase P, TP5, TP292 and PT332 reactions were assayed for inhibition by mature tRNA. Mature tRNA is a

competitive inhibitor of the inter-molecular RNase P reaction, but would not be expected to interfere with an intra-molecular reaction because the tethered substrate already occupies the active-site of the ribozyme. As shown in Figure 4 (Reaction conditions were the same as Fig. 2, with the addition of the indicated concentrations of non-radioactive competitor tRNA. The final concentration of enzyme and radioactive pre-tRNA in the native RNase P reactions was 2 nM in each reaction. Nat. P: native RNase P reaction), both the native RNase P RNA and TP5 reactions are inhibited by unlabeled competitor tRNA. In contrast, neither the TP292, nor the PT332 reactions were significantly affected by competitor tRNA concentrations as high as 1250 nM (ca. 50 times Km for native RNase P). This insensitivity of the TP292 and PT332 reactions to inhibition by tRNA is further indication that these reactions occur in cis.

Remodeling RNase P Substrate Specificity.

TP292 was modified to function as a sequence-specific endo-ribonuclease. Two such constructs, designated Endo.P1 and Endo.P2, were created by deleting 5' segments of the tethered tRNA. These constructs are illustrated in Fig. 5, which shows the secondary structures of the Internal Guide Sequence-encoding regions of Endo.P1 and Endo.P2 in detail, along with the sequences of their matched substrates. Arrows in Fig. 5 denote predicted sites of cleavage within the exogenous substrate RNAs. In Endo.P1 the 5' half of the acceptor stem was deleted (nt. 1-9), while in Endo.P2, this deletion was extended through the 5' half of the D stem (nt. 1-14). It was discovered that the tRNA sequences complementary to the deleted nucleotides (i.e. the 3' sides of the acceptor and D stems) were available to base-pair

with exogenous RNAs bearing complementary sequences. In effect, the tRNA segments of the tethered ribozymes constituted Internal Guide Sequences for recognition of exogenous RNA substrates.

The substrates tested with Endo.P1 and Endo.P2 consisted of 5' leader sequences joined to sequences complementary to the respective IGS sequences (Fig. 5). As shown in Figure 6, both Endo.P1 and Endo.P2 are capable of cleaving their complementary target RNA molecules. The optimal conditions for Endo.P cleavage are similar to those of TP292 self-cleavage (50°C, 2.5-3.0M ammonium acetate, 10 mM magnesium chloride). Under these conditions the Endo.P reaction rates were significantly slower than the rate of TP292 self-cleavage. Presumably, the rate of Endo.P cleavage is limited by the substrate binding and/or product release steps.

With both Endo.P1 and Endo.P2, cleavage occurs at the site predicted by the complementarity between the IGS and the substrate, as evidenced by the release of a product RNA that co-migrates with the 5' leader sequence generated in a native RNase P cleavage reaction. Thus, the substrate-binding specificity of RNase P was altered to create a novel ribozyme that accurately recognizes its substrate by Watson-Crick basepairing. In further studies, it was also discovered that both Endo.P1 and Endo.P2 are capable of cleaving RNA substrates with 3' ends that extend beyond the regions of IGS complementarity. Additionally, Endo.P1 functions as a true enzyme in catalyzing milutiple turnover reactions.

C. DISCUSSION

The applicants have designed and characterized several ribozymes derived from the catalytic RNA subunit of RNase P. The first set of ribozymes includes catalytic RNA molecules covalently linked to their substrate, pre-tRNA. Pre-tRNA-RNase P conjugates were constructed in which the substrate is positioned at or in close proximity to the active-site of the ribozyme. By tethering the pre-tRNA to circularly permuted RNase P molecules, the substrate could be linked to nucleotides of the ribozyme that are internal in the native RNA. Two sites of attachment, G292 and G332, were selected based on the association of these nucleotides with, respectively, the 3' and 5' ends of tRNA.

The resulting tethered molecules, TP292 and PT332, undergo self-cleavage *in vitro*, as judged by two criteria. First, the apparent rate constants of the TP292 and PT332 cleavage reactions do not vary over a 100-fold range of enzyme concentration; the rate of inter-molecular reactions would be expected to fall upon dilution of the enzyme. In contrast, both the native RNase P and TP5 reactions exhibited decreases in k_{app} upon dilution of reactants. Second, TP292 and PT332 cleavage reactions are unaffected by the addition of up to 1250 nM competitor tRNA (Km for the native RNase P-tRNA interaction is approximately 50 nM; Smith and Pace, 1993); the native RNase P and TP5 reactions were both strongly inhibited by equivalent levels of competitor tRNA. It can thus be concluded that the TP292 and PT332 cleavage reactions occur primarily through self-cleavage, rather than inter-molecular cleavage.

Kikuchi et al. (1993) recently reported that

tethered molecules similar to TP5 can undergo intramolecular cleavage to a limited extent. While the data herein do not absolutely exclude the possibility of TP5 self-cleavage, they do indicate that intermolecular cleavage predominates since the TP5 reaction rate is sensitive to both dilution and competition with competitor tRNA. An upper limit to the rate of TP5 self-cleavage (as opposed to intermolecular cleavage) can be estimated by the rate of cleavage measured at 0.02 nM TP5: 2.2×10^{-4} min^{-1} This rate is approximately four orders of magnitude slower than the rates measured for TP292 and PT332. Furthermore, the TP5 reaction often produced inaccurately cleaved products, an effect that was also reported by Kikuchi et al. (1993) in their analysis of TP5-like RNAs.

One rationale for designing self-cleaving RNase P derivatives was that such molecules should not be rate-limited by product release, as is the native RNase P reaction. If instead the chemistry of the reaction were rate-limiting for self-cleavage, then the conjugates could be useful tools for examining the effects of mutations or other perturbations on the rate of the chemical step of catalysis. As summarized in the Table, the first order rate constants of the TP292 and PT332 self-cleavage reactions are 1.5 and 1.2 min^{-1}, respectively (values measured at 0.02 nM). These rates compare favorably to the rate of the chemical step of native RNase P RNA, 2.3 min^{-1}, measured by Smith and Pace (1993) under single-turnover conditions at pH 6.0. TP292 and PT332 catalyzed reactions therefore are likely to be limited by the rate of phosphodiester bond cleavage. Thus, the active-site-tethered conjugates

should prove to be useful for studying catalysis by RNase P.

In addition to being fast compared to TP5 and native RNase P reactions, the TP292 and PT332 self-cleavage reactions are accurate. In both conjugates, greater than 99% of cleavage events occur at the appropriate phosphodiester bond, as judged by 3' terminal nucleotide analysis. Both TP292 and PT332 self-cleavage reactions also are efficient: reactions normally proceeded to 60-90% completion. The speed, accuracy and efficiency of TP292 and PT332 cleavage reactions all indicate that the substrate tRNA is tethered at or in close proximity to the active-site of RNase P in both conjugates. Furthermore, highly efficient cleavage is a required criterion for the application of in vitro selection schemes (Gold, et al., 1993; Szostak and Ellington, 1993) to the further analysis of RNase P function.

Preparation of Sequence-Specific Endonucleases

The TP292 conjugate was further modified to function as a sequence-specific endonuclease (Figs. 5 and 6). For Fig. 6, internally labeled substrate RNAs (Pre.P1 and Pre.P2) were incubated with or without their respective ribozymes (Endo.P1 and Endo.P2). pre-tRNA^{Asp} cleavage by native RNase P was included as a control of specific cleavage; cleavage of Pre.P1 and Pre.P2 is predicted to release a fragment identical to the 5' leader sequence of pre-tRNA. Reaction conditions: 60 min., 50°C, 16.5 mM PIPES, 44 mM Tris-Cl (pH 8.0), 0.1% SDS, 2.5 M ammonium acetate and 10 mM magnesium chloride. Reactions were 100 nM in both enzyme and substrate. P1: Pre.P1-Endo.P1 reaction. P2: Pre.P2-Endo.P2

reaction. N: Native RNase P + pre-tRNA^{ASP}. In each of the two configurations tested, the 3' half of the tRNA acceptor stem (as well as the D stem in construct Endo.P2) functioned as an IGS that bound exogenous RNA substrates. Both Endo.P1 and Endo.P2 cleaved substrate RNAs at the position predicted by the complementarity between their IGS sequences and the substrate RNAs. The absence of significant mis-cleavage indicates that Watson-Crick basepairing allows highly sequence-specific cleavage by these constructs. By altering the nucleotide sequence within the IGS one can design Endo.P ribozymes capable of cleaving any given single-stranded RNA.

While the invention has been illustrated and described in detail in the foregoing description, the same is to be considered as illustrative and not restrictive in character, it being understood that only the preferred embodiments have been shown and described and that all changes and modifications that come within the spirit of the invention are desired to be protected.

REFERENCES

The following references are indicative of the level of skill in the art and are hereby incorporated by reference in their entirity as if each had been individually incorporated by reference and fully set forth.

Altman, S. (1993). Proc. Natl. Acad. of Sci. USA. 90, 10898-10900.

Altman, S., Kirsebom, L. & Talbot, S. (1993). Faseb J. 7, 7-14.

Brown, J. W. & Pace, N. R. (1992). Nucleic Acids Res. 20, 1451-1456.

Burgin, A. B. & Pace, N. R. (1990). EMBO J. 9, 4111-4118.

Clark, J. M. (1988). Nucleic Acids Res. 16, 9677-9686.

Davies, R. W., Waring, R. B., Ray, J. A., Brown, T. A. & Scazzocchio, C. (1982). Nature. 300, 719-724.

England, T. E., Bruce, A. G. & Uhlenbeck, O. C. (1980). Methods in Enzymology. 65, 65-74.

Forster, A. C. & Altman, S. (1990). Science. 249, 783-786.

Frank, D. F., Roiha, H. & Guthrie, C. (1994). Mol. Cell. Biol. 14, 2180-2190.

Gold, L., Tuerk, C., Allen, P., Binkley, J., Brown, D., Green, L., MacDougal, S., Schneider, D., Tasset, D. & Eddy, S. R. (1993) In The RNA World (ed. Gesteland, R. F.&Atkins, J. F.), pp. 497-510. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Guerrier-Takada, C. & Altman, S. (1992). Proc Natl Acad Sci U S A. 89, 1266-1270.

Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N. & Altman, S. (1983). Cell. 35, 849-857.

Haas, E. S., Brown, J. W., Pitulle, C. & Pace, N. R. (1994). Proc. Natl. Acad. of Sci. USA. 91, 2527-2531.

James, B. D., Olsen, G. J., Liu, J. & Pace, N. R. (1988). Cell. 52, 19-26.

Kikuchi, Y., Sasaki-Tozawa, N. & Suzuki, K. (1993). Nucl. Acids Res. 21, 4685-4689.

Knap, A. K., Wesolowski, D. & Altman, S. (1990). Biochimie. 72, 779-90.

Milligan, J. F. & Uhlenbeck, O. C. (1989). Methods Enzymol. 164, 51-62.

Nishimura, S. (1979) *In transfer* RNA: Structure, Properties and Recognition (ed. Schimmel, P. R., Sîll, D.&Abelson, J. N.), pp. 551-552. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.

Nolan, J. M., Burke, D. H. & Pace, N. R. (1993). Science. 261, 762-765.

Pace, N. R. & Smith, D. (1990). J. Biol. Chem. 265, 3587-90.

Reich, C., Gardiner, K. J., Olsen, G. J., Pace, B., Marsh, T. L. & Pace, N. R. (1986). J. Biol. Chem. 261, 7888-7893.

Reich, C., Olsen, G. J., Pace, B. & Pace, N. R. (1988). Science. 239, 178-181.

Rossi, J. J. & Sarver, N. (1990). Trends in Biotech. 8, 179-183.

Segal, I. H. (1975) Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems, John Wiley & Sons, New York, N.Y.

Smith, D. & Pace, N. R. (1993). Biochemistry. 32, 5273-81.

Szostak, J. W. & Ellington, A. D. (1993) In The RNA World (ed. Gesteland, R. F.&Atkins, J. F.), pp. 511-534. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Tallsjî, A. & Kirsebom, L. A. (1993). Nucleic Acids Res. 21, 51-7.

Waugh, D. (1989). Ph.D. Thesis, Indiana University.

Waugh, D. S. & Pace, N. R. (1993). FASEB J. 7, 188-195.

Waugh et al. (1989). Science. 244, 1569-71.

Woese, C. R., Kandler, O., and M. L. Wheelis. (1990). Proc. Natl. Acad. Sci. USA. 87, 4576-4579.

WHAT WE CLAIM IS:

1. An endonuclease for sequence-specific, inter-molecular cleavage of a target nucleotide molecule, comprising:

an RNA molecule having nucleotides which provide the enzymatic activity of Ribonuclease P;

an oligonucleotide covalently bonded to the RNA molecule to form a conjugate, the oligonucleotide including a predetermined nucleotide sequence complementary to and available for hybridization with a nucleotide sequence of the target nucleotide molecule, wherein the conjugate is an endonuclease which inter-molecularly cleaves the target nucleotide molecule in a sequence-specific fashion.

2. The endonuclease of claim 1 wherein the target nucleotide molecule is RNA, the oligonucleotide is an oligoribonucleotide, and the predetermined nucleotide sequence includes a run of at least 5 nucleotides complementary to a sequence of the target RNA.

3. The endonuclease of claim 2 wherein the predetermined nucleotide sequence includes a run of 5 to 100 nucleotides complementary to a sequence of the target RNA.

4. The endonuclease of claim 1 wherein the RNase P is a bacterial RNase P.

5. The endonuclease of claim 2 wherein the target RNA is an RNA other than a pre-tRNA.

6. The endonuclease of claim 5 wherein the target RNA is a pathogenic or disease-causing RNA.

7. A method for sequence-specific cleavage of a target nucleotide molecule, comprising:

reacting the target nucleotide molecule with a sequence-specific endonuclease including:

an RNA molecule having nucleotides which provide the enzymatic activity of Ribonuclease P;

an oligonucleotide covalently bonded to the RNA molecule to form a conjugate, the oligoribonucleotide including a predetermined nucleotide sequence complementary to and available for hybridization with a nucleotide sequence of the target nucleotide molecule;

wherein the conjugate inter-molecularly cleaves the target nucleotide molecule in a sequence-specific fashion.

8. The method of claim 7 wherein the target nucleotide molecule is RNA, the ologionucleotide is an oligoribonucleotide, and the predetermined nucleotide sequence includes a run of at least 5 nucleotides complementary to a sequence of the target RNA.

9. The method of claim 8 wherein the predetermined nucleotide sequence includes a run of 5 to 100 nucleotides complementary to a sequence of the target RNA.

10. The method of claim 7 wherein the RNase P is a bacterial RNase P.

11. The method of claim 8 wherein the target RNA is an RNA other than a pre-tRNA.

12. The method of claim 11 wherein the target RNA is a pathogenic or disease-causing RNA.

13. A vector comprising a gene encoding a sequence-specific endonuclease of claim 1.

14. The vector of claim 13 which is a plasmid vector.

15. The vector of claim 13 which is a viral vector.

16. The vector of claim 13 wherein the target nucleotide molecule is RNA, the oligonucleotide is an oligoribonucleotide, and also comprising an RNA polymerase start sequence operably associated with the gene.

17. A gene encoding a sequence-specific endonuclease of claim 1.

18. The gene of claim 17 wherein the target nucleotide molecule is RNA, the oligoribonucleotide is an oligoribonucleotide, and wherein the gene is fused to an RNA polymerase start sequence.

19. A method for making a sequence-specific endonuclease to intermolecularly cleave a target nucleotide molecule, comprising:

creating a conjugate gene by covalently bonding a first gene encoding an oligonucleotide to a second, circularly permuted gene, the oligonucleotide including a predetermined nucleotide sequence complementary to and available for hybridization with a

nucleotide sequence of the target nucleotide molecule, and the circularly permuted gene encoding an RNA molecule having nucleotides which provide the enzymatic activity of Ribonuclease P;

cloning the conjugate gene into a vector for transcription; and

transcribing the conjugate gene to form a sequence-specific endonuclease for intermolecularly cleaving the target nucleotide molecule.

20. The method of claim 19 wherein the target nucleotide molecule is RNA and the oligonucleotide is an oligoribonucleotide.

AMENDED CLAIMS
[received by the International Bureau
on 03 November 1995 (03.11.95);
new claims 21-28 added;
remaining claims unchanged (1 page)]

21. The endonuclease of claim 1, wherein the RNAse P is a eubacterial RNAse P.

22. The endonuclease of claim 1, wherein said oligonucleotide is covalently bonded to the RNAse P at or near its active site.

23. The endonuclease of claim 21, wherein the oligonucleotide is covalently bonded at or near the active site of the RNAse P.

24. The endonuclease of claim 23, wherein the RNAse P is *E. Coli* RNAse P.

25. The method of claim 7, wherein the RNAse P is a eubacterial RNAse P.

26. The method of claim 7, wherein the oligonucleotide is covalently bonded at or near the active site of te RNAse P.

27. The method of clam 25, wherein the oligonucleotide is covalently bonded at or near the active site of the RNAse P.

28. The method of claim 26, wherein the RNAse P is *E. Coli* RNAse P.

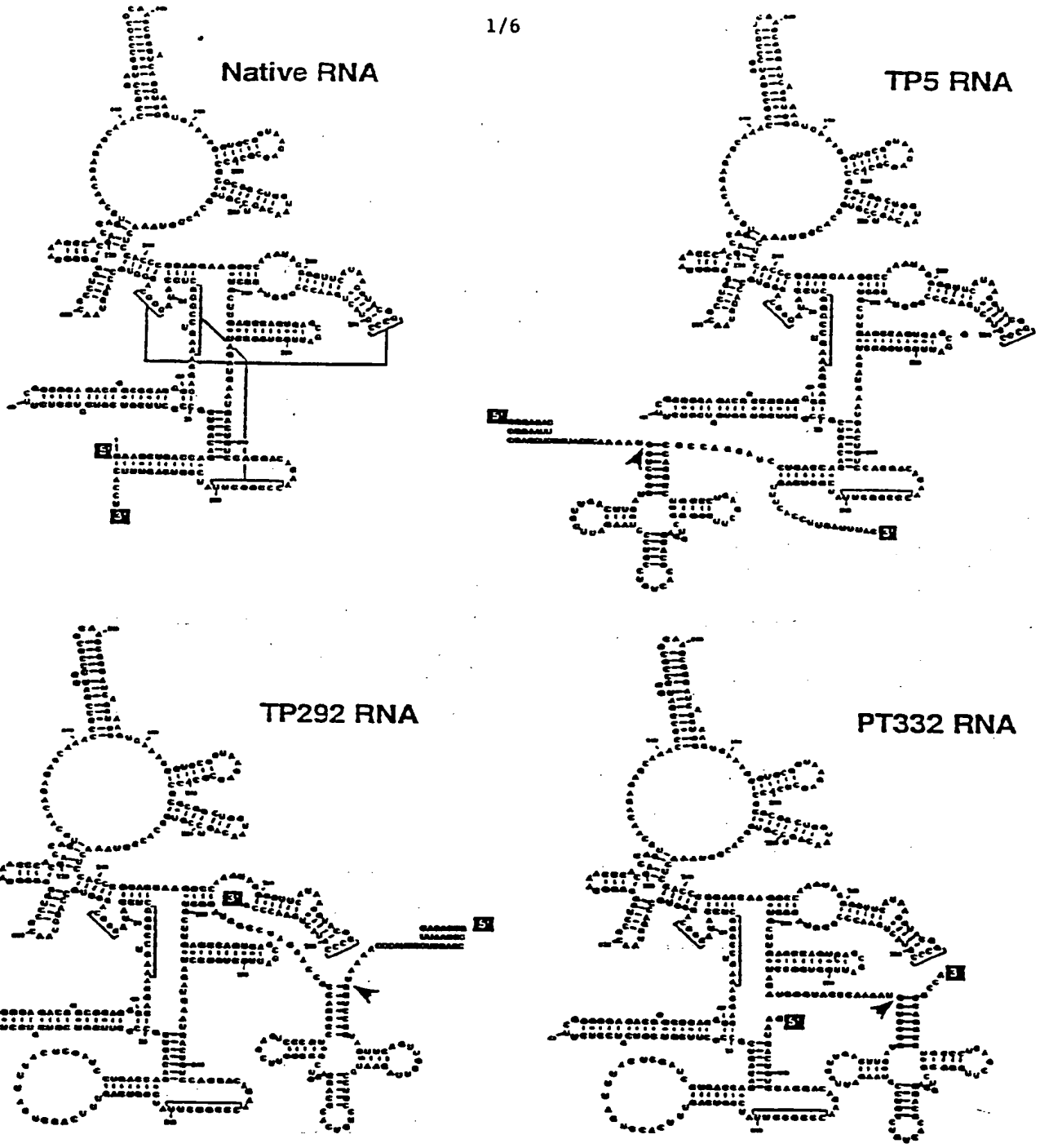


Figure 1

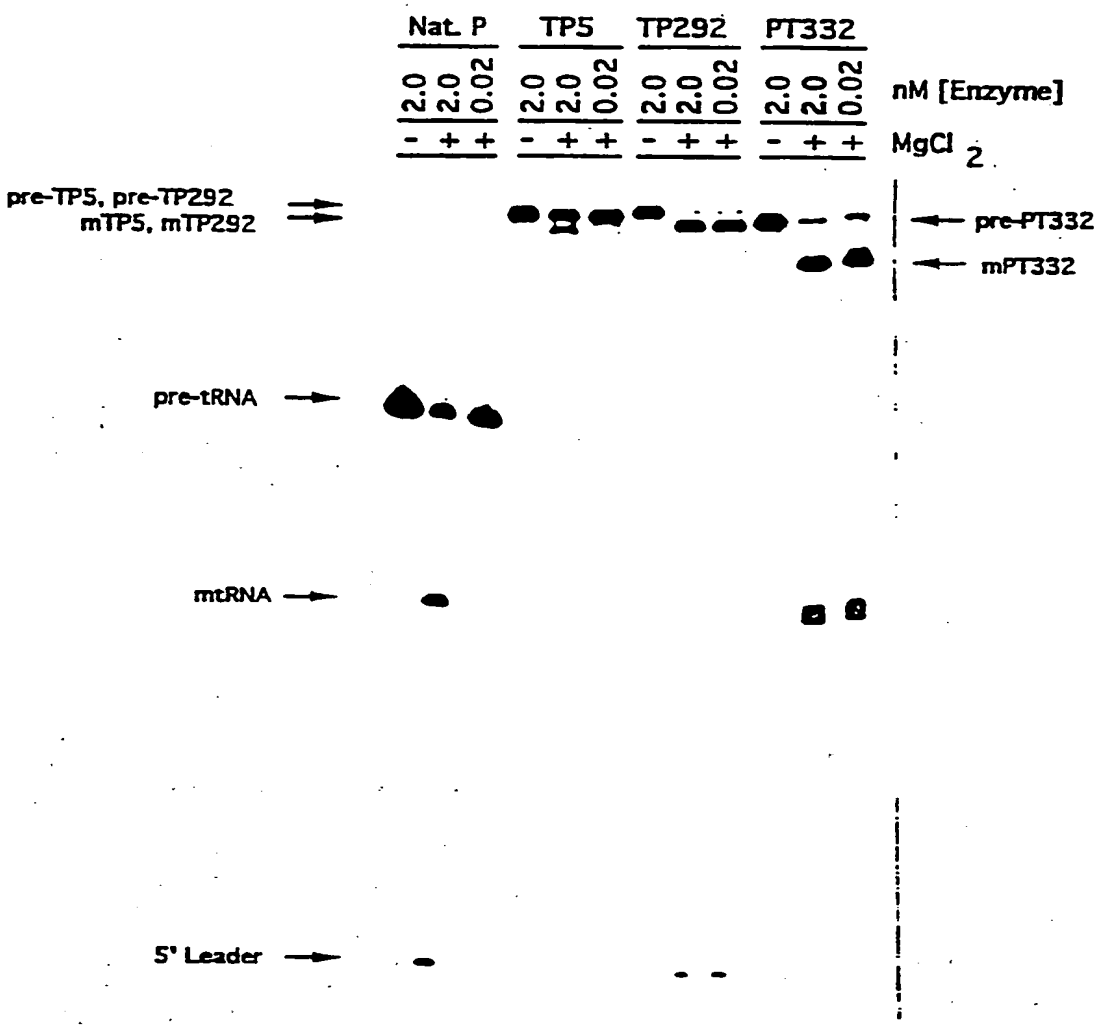

Nat. P
TP5
TP292
PT332
2.0 2.0 0.02
nM [Enzyme]
- + + MgCl 2
pre-TP5, pre-TP292
mTP5, mTP292
pre-PT332
mPT332
pre-tRNA
mtRNA
5' Leader

Figure 2

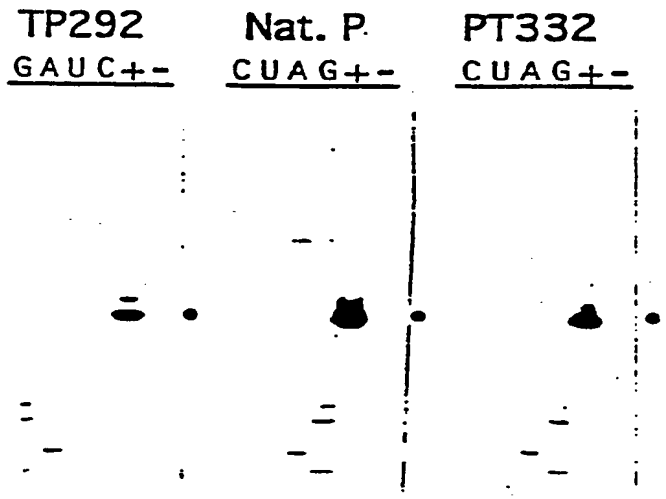


Figure 3.

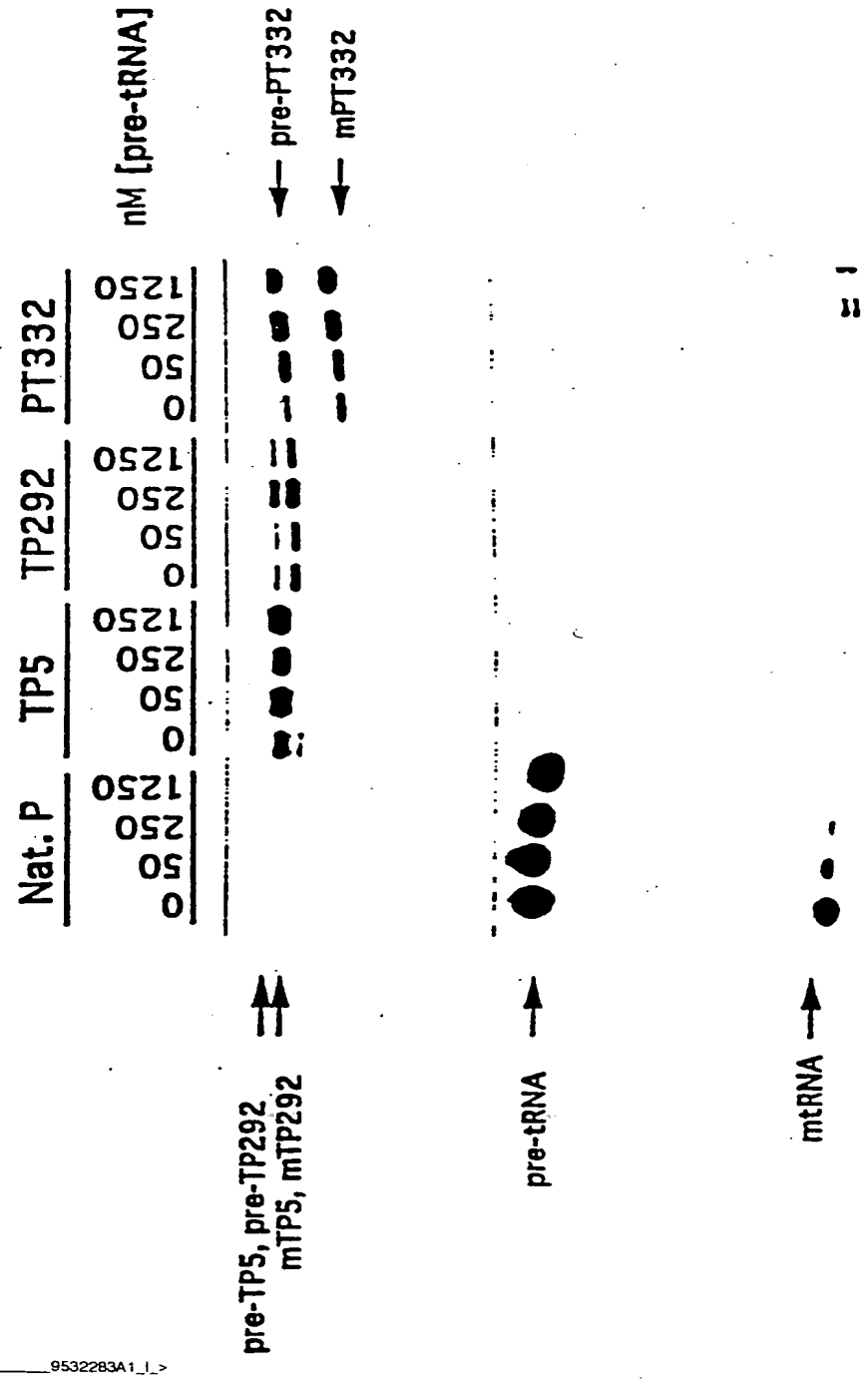


Figure 4

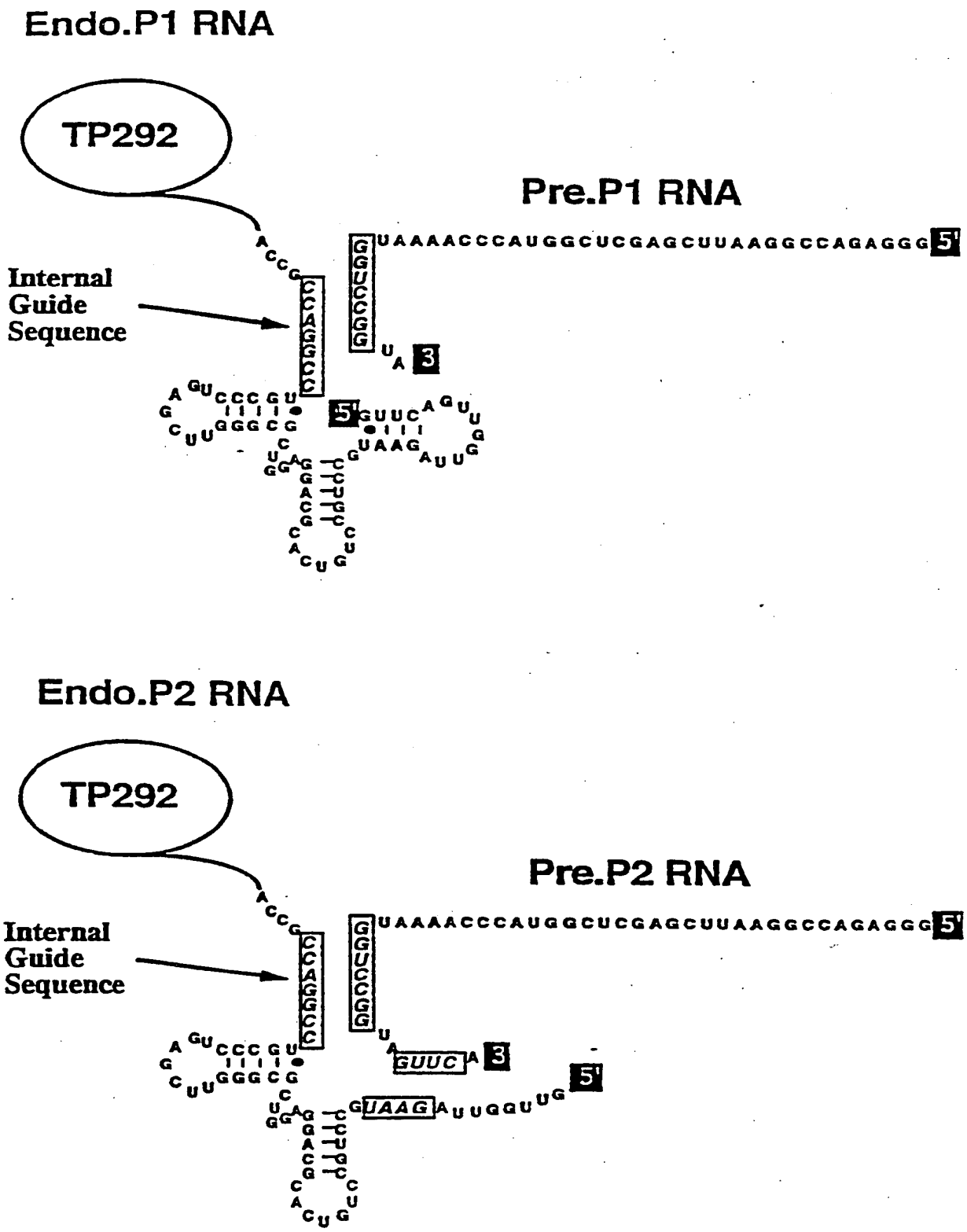

Endo.P1 RNA
TP292
Pre.P1 RNA
Internal Guide Sequence
UAAAACCCAUGGCUCGAGCUUAAGGCCAGAGGG
5'
3
Endo.P2 RNA
TP292
Pre.P2 RNA
Internal Guide Sequence
UAAAACCCAUGGCUCGAGCUUAAGGCCAGAGGG
5'
GUUC
3
5'
UAAG

Figure 5

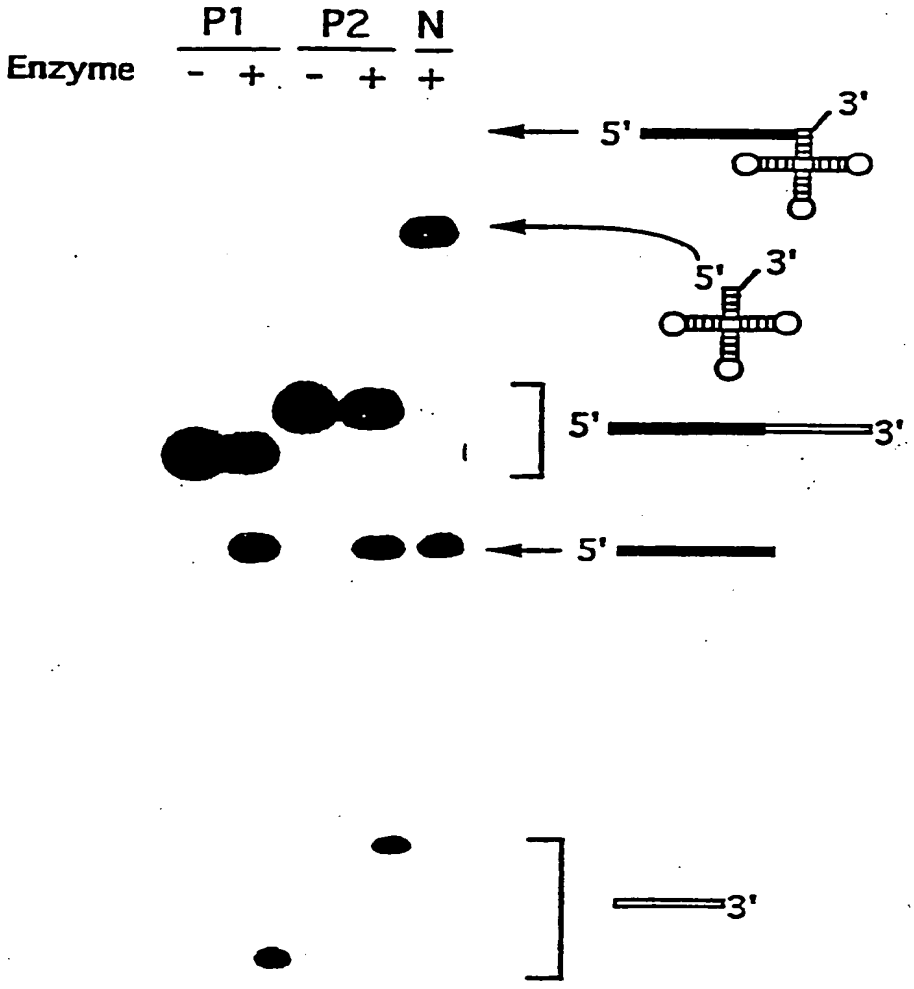


Figure 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/06519

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12N 15/09, 15/10, 15/11, 15/70; C12P 19/34
US CL :435/91.3, 172.3, 320.1; 536/23.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/91.3, 172.3, 320.1; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS
search terms: RNase P, ribozyme, endonuclease

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nucleic Acids Research, Volume 21, issued 1993, Kikuchi et al., "Artificial self-cleaving molecules consisting of a tRNA precursor and the catalytic RNA of RNase P", pages 4685-4689, see entire article.	1-20
Y	The FASEB Journal, Volume 7, issued January 1993, Altman et al., "Recent studies of ribonuclease P", pages 7-14, see entire article.	1-20
Y	Proceedings of the National Academy of Sciences USA, Volume 90, issued December 1993, Altman, "RNA enzyme-directed gene therapy", pages 10898-10900, see entire article.	1-20

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
25 AUGUST 1995

Date of mailing of the international search report
05SEP1995

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
PHILIP W. CARTER
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/06519

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cell, Volume 35, issued 1983, Guerrier-Takada et al., "The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme", pages 849-857, see entire article.	1-20
Y	Trends in Biotechnology, Volume 8, issued July 1990, Rossi et al, "RNA enzymes (ribozymes) as antiviral therapeutic agents", pages 179-183, see entire article.	1-20
Y	Proceedings of the National Academy of Sciences USA, Volume 89, issued April 1992, Li et al., "Targeted cleavage of mRNA *in vitro* by RNase P from *Escherichia coli*", pages 3185-3189, see entire article.	1-20
Y	Science, Volume 249, issued 17 August 1990, Forster et al., "External guide sequences for an RNA enzyme", pages 783-786, see entire article.	1-20